Electronic supplementary information to the communication:

Towards real time analysis of protein secretion from single cells

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Materials and methods

Strains and media

The *S. pombe* auxotrophic strain NCYC 2036 (*h⁻ ura4-D18*) was transformed with plasmid pJMN6 to yield the new strain JMN6.[1] This new strain is in the following denoted as eGFP (enhanced green fluorescent protein) strain/cell, while the parental strain NCYC2036 is referred to as wild type strain/cell. Briefly plasmid pJMN6 was constructed by inserting a cpy1 secretion signal[2] and a scFv-cDNA[3] into plasmid pREP42GFP-C.[4] In this construct transcription is regulated by the thiamin repressable *nmt41* promoter.[4]

Yeast cells were cultivated in 0.8 mL potassium phthalate (30 mM) buffered Verduyn medium[5] with 2% glucose and 0.1 g L^{-1} uracil in Eppendorf LidBac tubes using an Eppendorf Thermomixer Comfort (Eppendorf AG, Germany) at 800 rpm and 33 °C. The medium for the eGFP strain was uracil free to prevent segregative plasmid loss. For cultivation in the Envirostat, non-buffered Verduyn medium with a conductivity of 0.995 S m^{-1} was used.[6]

The microfluidic chip

The Envirostat, a single cell analysis platform allowing cultivation in a fully controlled environment, was used.[6] The core part of the Envirostat is a glass chip (Cytocon^{400}, Perkin Elmer, USA) and a power supply, which were developed by Fuhr's group.[7, 8] The bottom glass plane of the chip (thickness of 0.15 mm) is suited for fluorescence analysis. The chip bears seven dielectrophoretic elements for cell manipulation and trapping (Fig. supl.1 and next paragraph), controlled by the software Switch and a generator (Perkin Elmer, USA).[7] The overall channel height is 28 µm, and the width varies from 150 to 800 µm. The chip was connected *via* a newly developed world-to-chip interface.[9] Constant medium flow conditions were achieved by syringe pumps (World Precision Instruments Inc., USA). All solutions for *in-chip* usage were sonicated and filtered (0.2 µm pore size). Tubings, valves, and chip were sterilized after each experiment with a 40% ethanol solution. For further handling descriptions see Kortmann *et. al.*.[6]

In chip single cell cultivation, induction, and analysis

Yeast cells in the Envirostat were manipulated and trapped by negative dielectrophoresis (nDEP), as reported previously.[6] For inoculation, yeasts were harvested with a 2 µL syringe from an exponentially growing culture. Two hundred to 500 nanolitres of cell suspension (~2,000 cells mL^{-1}) were injected into the inlet flow. The cells were transported by a flow velocity of 180 µm s^{-1} (equal to 2 nL s^{-1}) to the cell trapping

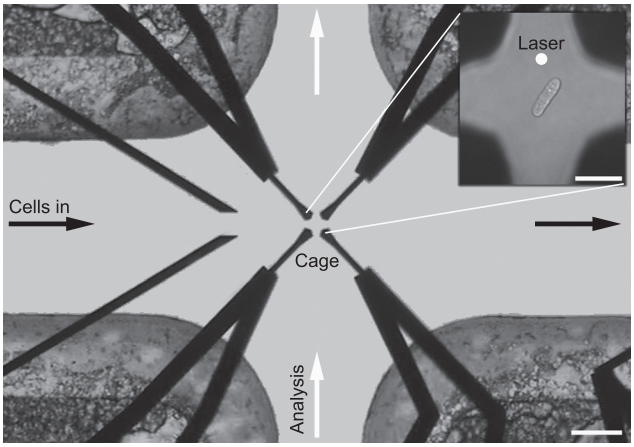

Fig. supl.1 Cell cultivation and analysis area in glass chip,[7] core part of the Envirostat. The black structures are electrode pairs; four pairs form the cage (cell trapping area) and two pairs on the left form a funnel structure. Cells entered the cage area through the main channel (indicated by the black arrows). For cell induction and during analysis the thiamine free medium entered from one side channel and left through the opposing side channel (indicated by the white arrows). Scale bar is 100 µm. The inset shows a trapped cell in the cage. The white dot illustrates the position of the laser during cell analysis. Scale bar is 10 µm.

area, the so called 'cage' (Fig. supl.1). Cells were trapped in the cage by nDEP (ROT mode at 2.6 V_{rms}, 7.5 MHz). The growth medium velocity during cultivation was 25 µm s^{-1} and the cultivation temperature 35 °C. Cells in the activated nDEP field are positioned and centered equidistant from the eight electrodes, thus floating contactless in the medium flow. To induce GFP secretion the Verduyn medium flow in the main channel was stopped and inductive (thiamin free) Verduyn medium was supplied trough a side channel (Fig. supl.1).

Optical setup

The Envirostat was mounted on the stage of an inverted microscope (Olympus IX71). For detection of eGFP, the blue line (488 nm) of an Argon ion laser (Laserlight, Berlin, Germany) is guided into the microscope, reflected by a dichroic mirror (405 DLPC, AHF Tübingen, Germany), and focused by a water immersion objective (UPlanApo 60x, NA 1.2, Olympus).[10] The laser focus diameter, determined by fluorescence correlation spectroscopy, was 0.72 µm. Exposure intensities of 103-105 W cm^{-2} were varied by placing neutral density filters into the beam path. Fluorescence light from the sample was collected by the same objective, passed a band pass filter (transparent for λ=500-550 nm, AHF, Tübingen, Germany), and was transferred via optical fibers (diameter 100 µm) to the detector (avalance photodiodes CD3017, EG&G, Vaudreil, Canada). The signal, *i.e.*, counted photons within a given integration time (here: 0.26 ms), was recorded

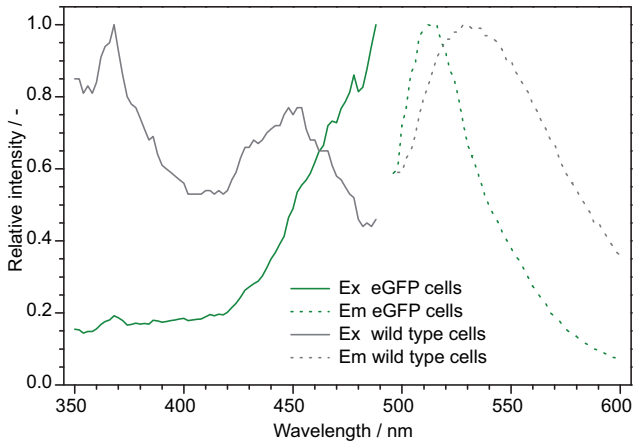

Fig. supl.2 Analysis of eGFP production by the eGFP *S. pombe* strain. Shown are the emission and excitation spectra of an eGFP cell culture and for comparison of a wild type cell culture.

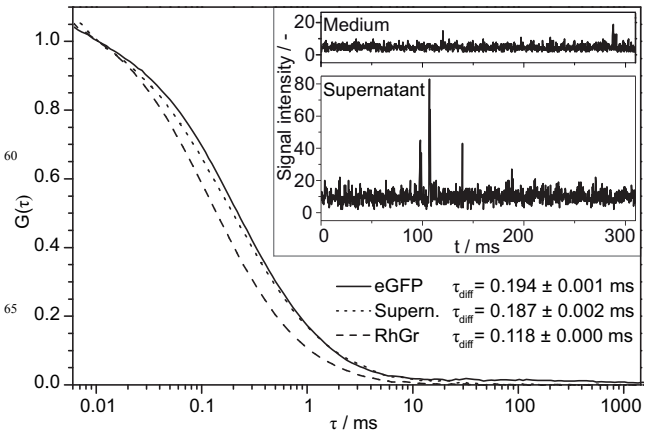

Fig. supl.3 Diffusion analysis by fluorescence correlation spectroscopy of purified eGFP and supernatant of an eGFP cell culture. The indicated diffusion time τ_{diff} is derived from the autocorrelation function.[11] Rhodamine Green (RhGr) was used for comparison. Inset: Detector signal from blank medium (top) and supernatant of an eGFP cell culture (bottom).

by a correlator card (ALV, Langen, Germany). The integration of 0.26 ms ensures that we obtain photons of one eGFP within one or maximum two integration times, as the eGFP dwell time was less than 0.2 ms in pure diffusion. Under the flowing conditions used here (25 µm s^{-1}) the dwell time is hardly shorter. During each experiment, data were collected every 15 min for 5 min. Additionally, the microscope is equipped with a mercury arc lamp and an EMCCD camera (iXon DV887, Andor Technologies, Belfast, Northern Ireland) for acquisition of fluorescence images.

Laser spot positioning

Diffusion of eGFP is a reason why close proximity measurement was chosen. Furthermore, CFD simulations revealed the position at which the highest concentrations of secreted molecules can be expected.[6] In the described experiments, the channel dimensions have a minor influence on the detection efficiency, i.e. the percentage of eGFP proteins detected of the total number of proteins that are secreted. On their way from the cells to the detection volume (ca. 3 µm), diffusion of GFP towards the channel walls is almost negligible, hence channel size does not influence the detection. Since detection was directly downstream of the cell, the size of the cell/cells compared to the size and position of the laser spot defines how many proteins can be detected. Particularly, careful position of the laser spot is required.

Results

Verification of eGFP production and secretion by the cells

Production of eGFP by the eGFP cells was verified by an emission-excitation scan of an eGFP culture. The spectra of the culture matched the eGFP characteristic (emission: 509 and excitation: 488) (Fig. supl.2). The spectra were taken with a microplate reader (Infinite 200, TECAN, Switzerland), for the emission spectrum the culture was excited with 465 nm, for the excitation spectrum emission at 520 nm was recorded (step size was 2 nm).

To test secretion of eGFP, we analyzed the cell free supernatant of the eGFP culture by confocal microscopy. The fluorescence trace of the supernatant showed bursts from secreted eGFP molecules. The mean signal intensity of the supernatant was twice as high as the mean intensity of the pure medium (Fig supl.3 inset). Moreover, the presence of secreted eGFP was verified via diffusion analysis using fluorescence correlation spectroscopy (FCS) (Fig. supl.3). The comparison of the autocorrelation function, G(τ), of the supernatant to those of pure eGFP (Biovision), and pure Rhodamine Green (RhGr) (Invitrogen) and the determination of the diffusion time, τ_{diff}, strongly indicated the existence of correctly folded eGFP in the supernatant (Fig. supl.3).

Consequently, the *S. pombe* produced and secreted active eGFP into the medium at concentrations that could be detected with standard laboratory equipment.

Cell viability test in the Envirostat

Prior to the eGFP detection tests in the chip, the viability of *S. pombe* under given in chip cultivation conditions was verified by monitoring cell growth (Fig supl.4).

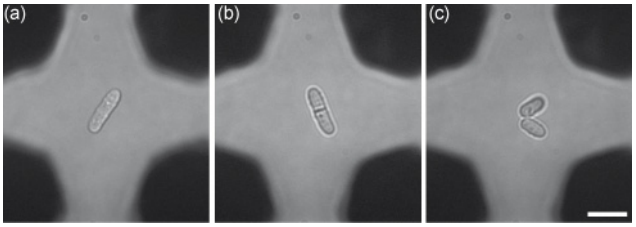

Fig. supl.4 The *S. pombe* growing in the nDEP cage, in (a) at cultivation start, in (b) after 50 min, and in (c) after 78 min. Cells were trapped and rotated by the nDEP field and were supplied by a constant Verduyn medium flow from the left. Black structures in the corners are the cage electrodes. Scale bar is 10 µm.

Particle displacement by beads

The existence of particle displacement, a phenomenon previously described for obstacles in a microfluidic chip,[12, 13] hypothesized to be present in the cell experiments was sustained by experiments using micro-beads of 11 µm in diameter. Micro-beads were used in order to exclude any cell bias. Indeed, particle displacement was clearly observed, as

the number of events downstream of single trapped micro-beads was reduced (Fig. supl.5), correlating well with results from wild type cells (Fig. 2). The absolute number of medium events (Fig. supl.5) differ from numbers in the cell

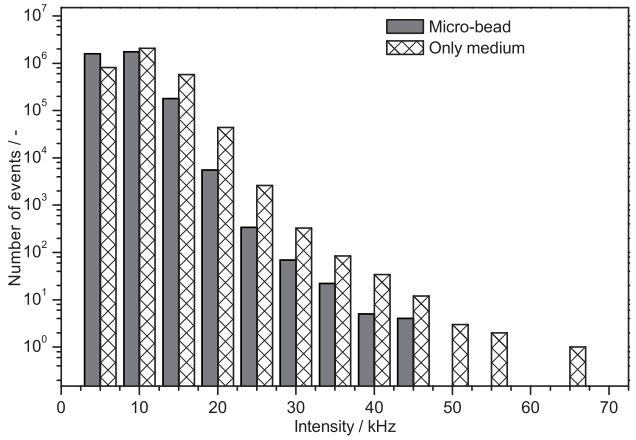

Fig. supl.5 Number of events in growth medium and in growth medium downstream of a micro-bead, with a diameter of 11 µm, detected in the chip by confocal microscopy. Each bar represents the summed number of events for 5 consecutive intensities of the mean value of 3 measurements.

experiments (Fig. 3 B and Fig. 4), highlighting the necessity to compare data from the very same experiment. Furthermore, the particel displacement results indicate that lateral laser positioning is crucial and close proximity to the cell will maximize signal to noise ratio.

Notes and references

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